Final Report for Screening of *Aspergillus niger* Strains for Enzyme Production in Sugar Beet Pulp Fermentations to Produce Fuel Ethanol

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Work Accomplished:

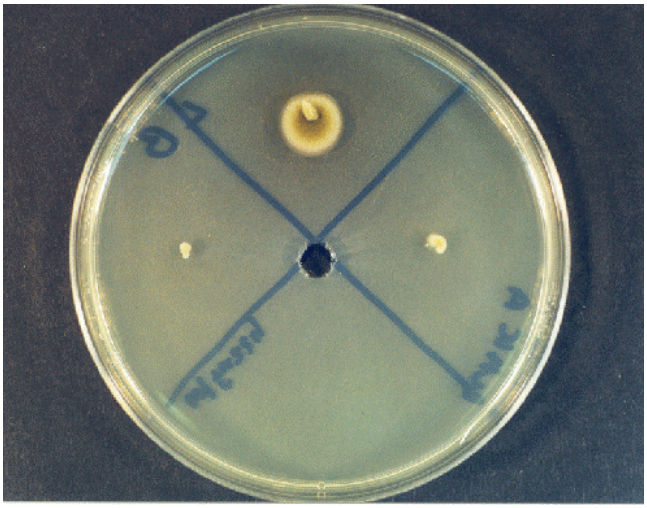
Certain microbes are capable of releasing enzymes that break down cellulose and pectin, the primary structural components of plants. These enzymes are commercially available for industrial applications, although they can be quite expensive. Studies have shown that strains of *Aspergillus niger* release cellulases and pectinases, enzymes that digest plant cell wall tissues. Approximately 40 cultures of *Aspergillus niger* were obtained from the USDA Culture Collection in Peoria, IL. Cultures were shipped in lyophilized form in glass vials and were reconstituted in potato dextrose agar and propagated in Doran's laboratory at CMU. Spore suspensions were standardized to an inoculum of 10^7 spores per ml of culture and inoculated onto medium containing carboxymethycellulose (CMC) and a second medium containing pectin. After incubation of 10 microliter to100 microliter volumes of spore suspension for varying time frames and temperatures, the radius of fungal growth was measured. The CMC plates were stained with congo red dye (0.1% w/v) which complexes with cellulose polymers longer than 5 units in length (Wood et al., 1988). Plates were destained with a 1 M solution of NaCl. Where CMC hydrolysis occurred, a clear zone or yellowish halo was present around the fungal growth. If no degradation of CMC occurred, the plate remained red after staining with Congo red and destaining with 1 M NaCl. Cultures were also screened for degradation of pectin. After incubation of various quantities of inocula for varying time frames and temperatures, the radius of fungal growth was measured on plates containing pectin. A 0.05% solution of ruthenium red flooded the plates for approximately one hour. Then plates were then washed with tap water. Pectin degradation was evidenced by a clear or light pink zone around the fungal growth. No pectin degradation was observed as no change in the appearance of the plate (Collmer et al., 1988). Cultures exhibiting the largest zones of clearing on the CMC and pectin medium were evaluated for enzyme production in liquid medium according to Methods in Enzymology Assays for Pectic Enzymes (Collmer et al., 1988

The best four strains (based on solid medium screening)were cultured for enzyme production in liquid medium (SMF, Solis-Pereira et al., 1993). Culture filtrates with the greatest level of pectin degradation were evaluated for degradation of sugar beet pulp. Carbohydrates released were evaluated by the Dinitrosalicylic acid assay for reducing sugars. Sugar beet pulp was utilized as a substrate for fungal enzyme depolymerization and used in fermentations with recombinant bacteria.. Pulp was obtained from a local processing plant (Monitor Sugar, Bay City, Michigan) and stored at -20°C until hydrolysis Recombinant ethanologenic bacteria were provided by Dr. Lonnie O. Ingram (Univ. of Fl) and cultures are stored in 40% glycerol at -20°C and maintained on complex medium containing 40 mg/ml chloramphenicol. Commercial enzyme preparations were needed with the fungal culture filtrates. Supplementation with low levels of commercial enzymes was required for concentrated (40 g/L) ethanol streams. Pectinex Ultra and Celluclast were supplied by NOVO Nordisk Labs, Franklinton, NC. SSF experiments were conducted in modified 1200-ml fermentation vessels containing 450-ml working volume of substrate essentially as described (Beall et al., 1991).

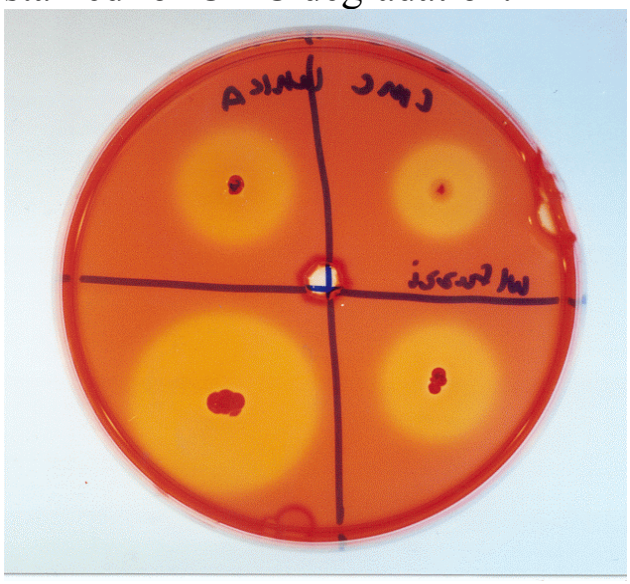
Fermenters contain a pH probe, CO_2 vent, sampling needle, and port for the addition of base. Magnetic stirrers were located beneath the water bath and maintained at 100 rpm. Stirring was provided by a Teflon-coated magnet. The pH was maintained using a Jenco model 3671 pH controller (Whatman Lab Sales, Hillboro, OR) which activated a solenoid valve (Rainin Instrument Co., Woburn, MA). Temperature regulation was maintained by a thermoregulator. Initial experiments were conducted using process parameters determined by use of sugar cane bagasse and *Klebsiella oxytoca* strain P2 (Doran and Ingram, 1993; Doran et al., 1994). Experiments utilized recombinant ethanologenic *Escherichia coli* and commercially available enzyme preparations described previously to determine the lowest level of enzyme loading necessary to reach an ethanol concentration that will permit cost efficient recovery. Cells were harvested by centrifugation and used for SSF experiments at an initial cell density of 330 mg dry wt./L. The pH and base consumption were recorded. For ethanol analysis, 2 ml samples were removed and clarified by centrifugation. Ethanol content was determined by gas-liquid chromatography essentially as previously described (Ingram et al., 1987). The final phase of the project, large scale fermentations using the culture filtrates and sugar beet pulp, was not completed due to hospitalization of the PI. I was hospitalized for 3 months for preterm labor and gave birth to twin girls in June. During my hospitalization I was quite ill from the medications and had to take medical leave.

Light microscope photograph of *Aspergillus niger*

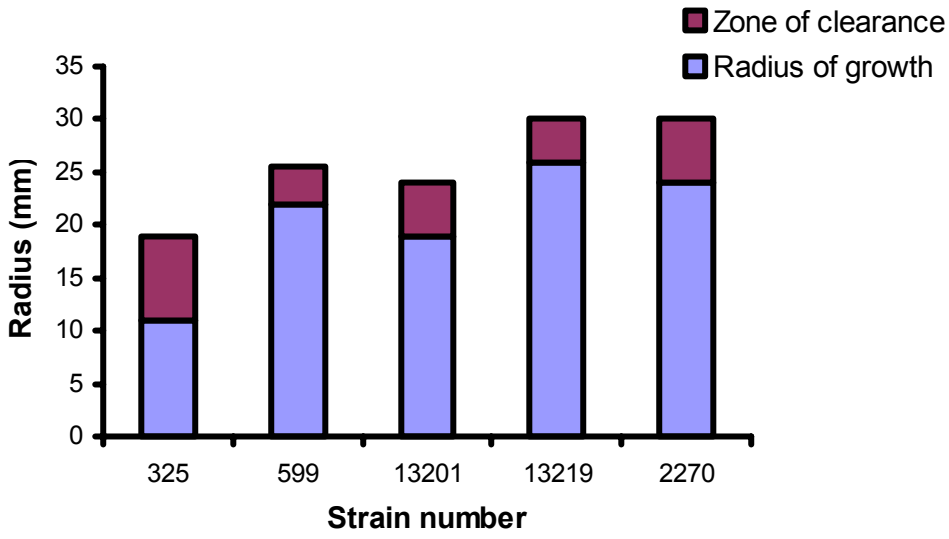
Petri plate containing 100 µl of 10^7 spore suspensions of 4 *A. niger* strains.



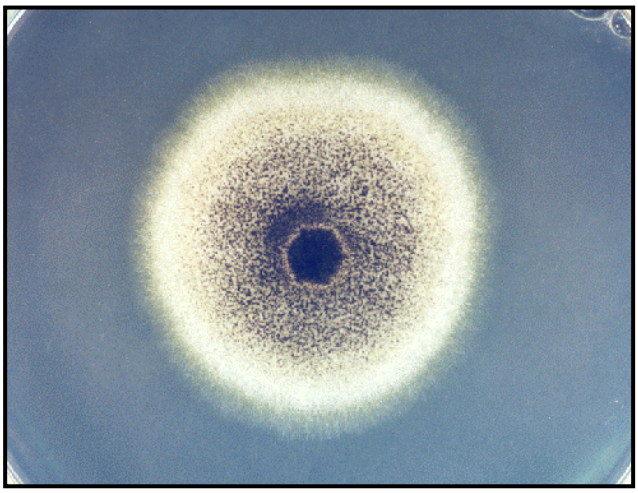
Petri plate containing 100 µl of 10^7 spore suspensions of 4 *A. niger* strains stained for CMC degradation.



Results of *A. niger* screening on CMC medium.



Petri plate containing pectin and 100 µl of a 10^7 spore suspension of *A. niger* strain 325.



Petri plate containing pectin and 100 µl of a 10^7 spore suspension of *A. niger* strain 325 stained with ruthineum red.

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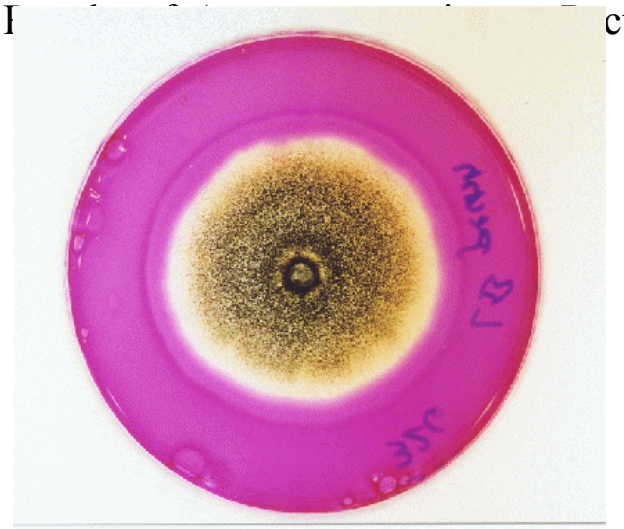

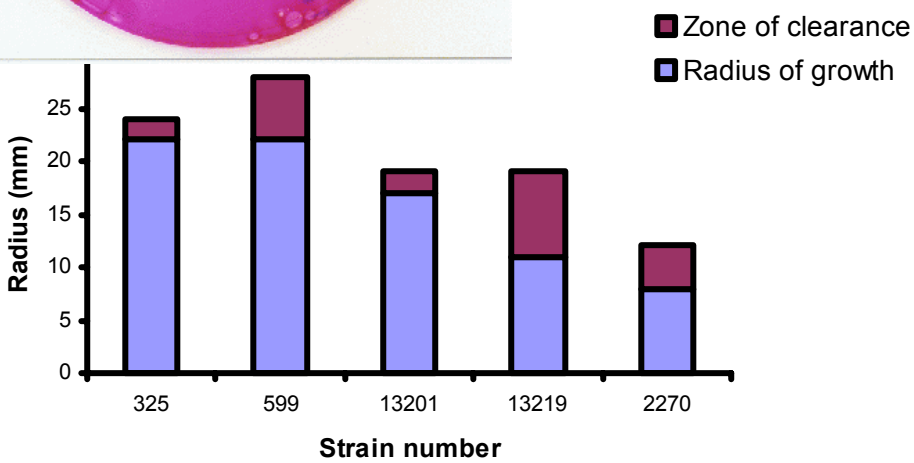

Executive Summary

Using ethanol as an alternative to petroleum based fuels significantly reduces automobile emissions and requires few alterations in current vehicle technology to be implemented. Sugar beet pulp (SBP), an agricultural co-product of the table sugar industry, shows promise as a substrate for microbial fermentations to ethanol. Sugar beet pulp is rich in cellulose, hemicellulose, and pectin that serve as carbohydrate sources for microbial fermentative metabolism. However, these carbohydrate polymers must be degraded to their simple sugar subunits in order to be used by fermenting microorganisms that often lack some or all of the enzymatic activities. Commercially available fungal enzymes digest SBP, although they are an expensive component of the bioconversion process. Experiments were conducted to determine the amount of ethanol produced from various combinations of commercially available fungal enzymes with genetically engineered ethanologenic *E. coli* as biocatalysts. In an effort to reduce the amount of commercially available fungal enzymes needed to produce over 30 g ethanol/liter, the pulp was predigested with crude culture filtrates from selected strains of *Aspergillus niger* supplied by the USDA Laboratory Agricultural Service Culture Collection. Over 40 strains from the USDA culture collection were screened for cellulase and pectinase activities on solid media. A 10^7 spore suspension was inoculated onto Luria Bertani (LB) agar containing carboxymethylcellulose (CMC), and LB plates with a pectin overlay. After 5 days of incubation at 22^{o}C, CMC plates and LB-pectin plates were stained with Congo red and Ruthenium red, respectively. After destaining with NaCl and distilled water, zones of clearance were visible around the fungal growth as a result of enzymatic activities. Strains with the largest zones of clearance with respect to their radii of fungal growth were selected to be utilized in biomass fermentations using sugar beet pulp. Crude culture filtrates contained approximately 100 fold less activity than commercially available enzyme preparations of Celluclast and Pectinex provided by Novo Nordisk, Inc. Using the crude culture filtrate in sugar beet pulp fermentations reduced the need for commercially available fungal preparations by 10%. These crude culture filtrates are being evaluated for specific enzymatic activities including pectate lyase, pectin methyl esterase, and carboxymethylcellulose activites. Concentrating the culture filtrate and adding more units of activity should enhance the overall sugar beet pulp- to -ethanol process.